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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12P 41/00

A1

(11) International Publication Number: WO 86/ 07386

(43) International Publication Date: 18 December 1986 (18.12.86)

(21) International Application Number: PCT/DK86/00061

(22) International Filing Date: 10 June 1986 (10.06.86)

(31) Priority Application Number: 2616/85

(32) Priority Date: 11 June 1985 (11.06.85)

(33) Priority Country: DK

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: PROCESS FOR PREPARING OPTICALLY ACTIVE, ORGANIC COMPOUNDS

(57) Abstract

Optically active amino acids or amino acid amides can be prepared by converting an amino nitrile using an enantioselective nitrilase.

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PROCESS FOR PREPARING OPTICALLY ACTIVE, ORGANIC COMPOUNDS

Background of the invention

The present invention relates to a process for preparing optically active amino acids. More specifically, this invention relates to a process for preparing a single enantiomeric form of an optically active amino acid or amino acid amide which comprises treating an aqueous solution of the enantiomeric mixture of the amino nitrile analog of the amino acid with an enantioselective nitrilase and thereafter recovering the resulting optically active amino acid or amino acid amide.

Optically active amino acids constitute a class of organic compounds of great industrial interest. The naturally occurring amino acids are thus applied industrially on a large scale as food and feed additives and, in recent years, several amino acids not found in nature and in the following referred to as unnatural amino acids have also found extensive use, for example, as constituents in various pharmacological compositions or as intermediates for organic synthesis of optically active compounds.

Due to their molecular structure, most amino acids can occur in two distinct forms differing in respect to the so-called chirality of the amino acid molecule. These two forms of an amino acid which, on the molecular level, are mirror images of one another are usually denoted as the D- and the L-form of the amino acid. Most amino acids found in nature are of the L-configuration and it is essential, therefore, that amino acids used as food and feed additives are also of the L-configuration since the corresponding D-forms or isomers cannot be metabolized by living cells and will interfere with normal cell metabolism and cell function. This ability of the D-amino acids can, however, also be utilized to advantage, for example, by incorporating such unnatural isomers of amino acids into pharmacologically active compounds, the activity of which may be due to or enhanced by a moiety of unnatural chirality in its molecular structure. In such instances, it is essential that the amino acid used only is of the unnatural configuration since the presence of

molecular species carrying the natural configuration will, in such instances, excert a deleterious effect on the biological activity of the compound in question.

Because of the wide use of natural as well as of unnatural amino acids it is, in general, highly desirable to have available optically pure, i.e., enantiomerically pure, amino acids of the natural as well as of the unnatural configuration for a wide variety of industrial applications of amino acids while, on the contrary, mixtures of the D- and L-forms of amino acids, the so-called racemates, are of limited industrial interest only.

The desire to provide en excess amount of one enantiomer in preparations of amino acids is reflected in the methods currently used for industrial production of such compounds. Most amino acids used as food and feed additives are thus produced by microbial fermentations which, due to the very nature of the microorganisms, give rise solely to amino acids of the natural configuration. Also, enzymes derived from microorganisms or other living matter have been used for the production of amino acids which, in such instances, derive their chirality from the chirality of the applied enzyme.

An example of an enzymatic method which has been used for preparation of optically active amino acids is described in U.S. patent specifications Nos. 4,080,259 and 3,971,700. The process disclosed in these patents can be illustrated in the following Scheme 1:

$$Ph\text{-}CHO + HCN + NH_3 \rightarrow$$

$$Ph\text{-}CH(NH_2)\text{-}CN \rightarrow$$
(D,L)

$$Ph\text{-}CH(NH_2)\text{-}CONH_2 \xrightarrow{\text{amino peptidase}}$$
(D,L)

$$Ph\text{-}CH(NH_2)\text{-}COOH + Ph\text{-}CH(NH_2)\text{-}CONH_2$$
(L) (D)

wherein Ph represents, for example, phenyl.

As indicated, an enzyme, i.e., an amino acid amidase, a so-called amino peptidase, is utilized for converting amino acid amides into the corresponding amino acids. As appear from Scheme 1, the amino acid amides used in the process illustrated are made available by chemical synthesis from achirale starting materials via racemates of amino acid nitriles, the consequence being that the amino acid amides used in the process described are racemic mixtures. The enzyme used in the process is, however, chirale and, therefore, capable of distinguishing between the two isomeric forms of the amino acid amide. As a consequence, the amino acids generated in the course of the amino peptidase catalyzed reaction are of the L-configuration while the amino acid amides remaining in the reaction mixture after completion of the enzymatic conversion are of the D-configuration. These two, chemically distinct species, can be separated by conventional methods and the enantiomeric pure amino acid amides thus obtained can subsequently be hydrolyzed by chemical means to provide optically pure D-amino acids. The method disclosed in the above U.S. patent specifications serves, therefore, as a means for the preparation of optically pure L- as well as D-amino acids.

The use of enzymes for the conversion of amino nitriles into the corresponding amino acid amides is a feasible process which, however, does not so far offer any

phenyl optionally substituted by one or more of the following substituents: hydroxy, amino, halogen, carboxy or lower alkoxy; and X represents hydroxy or amino; or salts thereof.

Hence, the starting material is an amino nitrile of the general formula II

$$R\text{-}CH(NH_2)\text{-}CN \qquad (II)$$

wherein R is as defined above, or a salt thereof..

Examples of the substituent designated R are as follows: methyl, isopropyl, secundary butyl, phenyl, p-hydroxyphenyl, benzyl, 1-hydroxyethyl, mercaptomethyl, methylthiomethyl, benzyloxy and phenoxymethyl. Preferably R is indolyl or benzyl optionally substituted by one or more of the following groups: hydroxy, amino and/or lower alkoxy.

Herein the term lower alkyl designates alkyl containing less than 8, preferably less than 5, carbon atoms. Similarly, lower alkoxy contains less than 8, preferably less than 5, carbon atoms.

The enzymatic process may, according to this invention, be carried out, for example, in a batch-wise fashion by stirring a mixture of the nitrilase and the amino nitrile in an aqueous solution under control of the pH value and temperature of the reaction mixture. The reaction temperature may be between the freezing point of the reaction medium and about 65°C, preferably between 20 and 45°C, most preferred about 37°C. If desired, organic solvents can be utilized to increase the solubility of the reactants, such solvents being, for example, alcohols such as ethanol, methanol, isopropanol or tertiary butanol or organic solvents such as dioxane, N,N-dimethylformamide, dimethylsulfoxide or hexamethylphosphorous triamide. The reaction may also be carried out in a two-phase system using a suspension of reactants or two immicible solvents like, for example, water and a hydrocarbon such as hexane or cyclohexane.

The nitrilase applied in the process of this invention may be a purified enzyme, a crude enzyme solution, microbial cells exhibiting the desired activity or a

homogenate of cells. If required, the enzyme may be used in an immobilized state or in a chemically modified form to ensure a good stability and reactivity of the applied enzyme under the reaction conditions utilized.

The process of this invention can be carried out at neutral or at an alcaline pH value to ensure rapid interconversion of one of the two enantiomeric forms into the other of the two enantiomeric forms of the amino nitriles used as starting material in the enzymatic process. This interconversion can also take place at a pH value below 7 or it can be ensured by applying an amino nitrile racemase. Hence, preferentially, the pH value is from about 6 to about 13.

As mentioned above the nitrilases used by the process of this invention are enzymes exhibiting a different activity towards the two enantiomeric forms of amino nitriles. Preferably, nitrilases exhibiting a strong selectivity towards one of these enantiomers are used since it is usually desired that the amino acids or amino acid amides prepared by the process of this invention contain a large excess of one of the two enantiomers. In a preferred embodiment of this invention, the excess of one of the two enantiomeric forms of the amino acid or amino acid amide is greater than 25%. Accordingly, it is preferable to test nitrilases prior to use for conversion of a given amino nitrile. This test can be carried out, for example, by exposing the amino nitrile in question to the enzyme preparation and by, subsequently, isolating, after conversion of a small amount of the amino nitrile, the amino acid amide and/or amino acid formed, for example, by high pressure liquid chromatography, and by analyzing the optical purity of the isolated compounds. Preferably, this test is carried out at various degrees of conversion of the applied amino nitrile.

The enzymes for use in the process of this invention may be isolated from microorganisms, plants or animals. Preferably, however, enzymes of microbial origin are utilized, such microorganisms being bacteria, fungi or other microorganisms.

Examples of microbial species producing nitrilases are as follows: Species of Pseudomonas, Gluconobacter, Acetobacter, Achromobacter, Acinetobacter, Citrobacter, Enterobacter, Erwinia, Escherichia, Klebsiella, Proteus, Serratia, Yersinia, Aeromonas, Vibrio, Staphylococcus, Streptococcus, Clostridium, Leuconostoc, Cellulomonas, Microbacterium, Propionibacterium, Mycobacterium, Streptomyces, Chaetomella, Septoria, Diplodia, Phoma, Conothyrium, Myrothecium, Pestalotia, Melanconium, Epicoccum, Penicillium, Aspergillus, Sepedonium, Fusidium, Oidiodendron, Cephalosporium, Scopulariopsis, Paecilomyces, Verticillium, Tricothecium, Pullularia, Monotospora, Cladosporium, Helminthosporium, Chrysosporium, Rhodotorula, Kloeckera, Geotrichum and preferably Fusarium, Agrobacterium, Arthrobacter, Alcaligenes, Shigella, Peptococcaceae, Pseudomonadaceae, Cytophaga, Bacteroidaceae, Butyrivitrio, Selenomonas, Zymomonoes, Chromobacterium, Flavobacterium, Micrococcus, Pediococcus, Bacillus, Lactobacillus, Brevibacterium, Thermus, Corynebacterium, Hyphomicrobium, Bacteridium, Actinomycetales, Rhizopus, Mucor, Candida, Saccharomyces, Nocardia, Rhodococcus, Stenphylium and Torylopsis, strains of Agrobacterium radiobacter, Pseudomonas aeroginosa, Pseudomonas fluorescens, Pseudomonas putida, Corynebacterium nitrilophilus, Corynebacterium pseudodiphteriticum, Nocardia rhodochrous, Escherichia coli, Neurospora crassa, Lathyrus sylvestris, Lathyrus odoratus, Vicia villosa, strain A4 (deposited at Laboratory of Microbiology (hereinafter designated LMD), the Netherlands, under No. LMD 79.2), strains N-771, N-774 and N-775 (deposited at Fermentation Research Institute (hereinafter designated FRI), Japan, under No. 4445, 4446 and 4447, respectively) and strains R 332 (deposited at Centraalbureau voor Schimmelcultures (hereinafter designated CBS), the Netherlands), R 340 (CBS No. 495.74), R 341 (CBS No. 496.74), A 111 (CBS No. 497.74), B 222 (CBS No. 498.74), A 112, A 13, A 141, A 142, B 211, B 212, B 221, C 211 (CBS No. 499.74), R

21, R 22, R 311, R 312 (CBS No. 717.73) and R 331 stated in Table I in U.S. patent specificaton No. 4,001,081 which is hereby incorporated by reference.

The desired amino acid amide or amino acid is isolated from the reaction mixture in a manner known per se, for example, by precipitation, optionally after adjustment of the acidity, or evaporation.

The features disclosed in the foregoing description and in the following examples and claims may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

The process of this invention will be further illustrated by the following examples which, however, are not to be construed as limiting. The examples illustrate some preferred embodiments.

Example 1

Preparation of optically active L-leucine amide

A preparation of an enantioselective aminonitrile hydratase was prepared by cultivating nitrilase producing strain No. 311 (deposited in May 1986 at the National Collection of Industrial Bacteria (NCIB) under the number NCIB 12256) in a modified M9 medium (c.f. Maniatis et. al., Molecular Cloning, A Laboratory Manual, CSH, 1982) containing 1% glucose, 0.05% yeast extract and 0.5% acetonitrile as substitute for ammonium chloride. The biomass generated was harvested after three days of growth at 37°C, washed thoroughly with phosphate buffer (0.1 M, pH 7) and finally stored as a suspension in said buffer. This suspension was used as the enzyme solution in the following examples.

A solution of racemic leucine aminonitrile was prepared in the following manner:

Ammonium chloride (0.032 mol) in 5.5 ml of water was added at room temperature to a solution of 3-methyl-butanal (0.031 mol) in 2.2 ml of water. After 30 minutes, the mixture was cooled to 0°C and a solution of sodium cyanide (0.031 mol) was added. The resulting mixture was then stirred for one hour at 0°C and then for 12 hours at room

temperature. Finally, the solution was diluted with phosphate buffer (0.1 M, pH 7) to a final concentration of the aminonitrile of 120 mM.

Enzymatic hydrolysis of the aminonitrile was subsequently performed by adding 0.1 ml of enzyme solution per 0.3 ml of the solution of the aminonitrile, stirring of the resulting mixture for 1 hour, removing the enzyme by centrifugation, and finally adsorbing the product and eluating it from an ion-exhange resin. The amide isolated in this fashion was found to contain an enantiomeric excess of the L-amide of 40%.

Example 2

Preparation of optically active L-leucine

A solution of leucine amino nitrile was made and treated with the enzyme solution described above in a manner analogous to that described in Example 1. At intervals during the enzymatic hydrolysis, the enzyme was removed by centrifugation after which pH of the reaction mixture was adjusted to 11 by addition of a 2 M sodium hydroxide solution. After 15 minutes, the pH of the reaction mixture was adjusted to its initial value and mixed with the biocatalyst. This procedure was carried out 5 times during a total reaction period of 6 hours after which conversion of the aminonitrile into the amino acid was complete as determined by thin layer chromatography. The amino acid was then isolated by ion-exchange chromatography and found to contain an enantiomeric excess of 35%.

Example 3

Preparation of optically active L-valine amide

L-valine amide was prepared from isobutyraldehyde in a manner analogous to that described in Example 1. The enantiomeric excess of the L-amide in the reaction mixture was found to be 35%.

Example 4

Preparation of optically active L-valine

L-valine was prepared from isobutyraldehyde in a manner analogous to that described in Example 2. The enantiomeric excess of the L-amino acid was found to be 30%.

C L A I M S

1. A process for preparing an amino acid or amino acid amide which comprises treating a solution of an enantiomeric mixture of the corresponding amino nitrile with an enantioselective nitrilase and subsequently recovering the resulting optically active amino acid or amino acid amide.

2. A process, according to Claim 1, characterized in preparing optically active amino acids or amino acid amides of the general formula I

$$R\text{-}CH(NH_2)COX \qquad (I)$$

wherein R represents indolyl; benzyl; benzyloxy; lower alkyl optionally substituted by hydroxy, mercapto, amino, halogen, phenyl, phenoxy, benzyl or lower alkylthio; or phenyl optionally substituted by one or more of the following substituents: hydroxy, amino, halogen, carboxy or lower alkoxy; and X represents hydroxy or amino; or salts thereof.

3. A process according to Claim 1 or 2, characterized in preparing an amino acid or amino acid amide of L-configuration.

4. A process according to any one of the preceding claims, characterized in preparing the enantiomeric amino acid or amino acid amide in an excess of at least 25%.

5. A process according to any one of the preceding claims, characterized in effecting the treatment at a pH value from about 6 to about 13.

6. A process according to any one of the preceding claims, characterized in that the conversion is effected in the presence of an amino nitrile racemase.

7. A process according to any one of the preceding claims, characterized in using a reaction temperature of from about 20 to about 45°C, preferably about 37°C.

8. A process according to any one of the preceding claims, characterized in that the conversion is effected in an aqueous medium optionally containing an alcohol, dioxane, N,N-dimethylformamide, dimethylsulfoxide or hexamethylphosphorous triamide.

9. A process, according to any of the preceding claims, characterized in using a nitrilase of microbial origin, preferably of bacterial origin.

10. A process according to claim 9 characterized in using an aminonitrile hydratase with enzymatic properties substantially identical with those of the aminonitrile hydrotase obtained by cultivation of Strain No. 311 deposited at the National Collection of Industrial Bacteria (NCIB) under number NCIB 12256 or a mutant thereof.

11. The use of an enantioselective nitrilase for the conversion of an aminonitrile into the corresponding optically active amino acid or amino acid amide.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/DK86/00061

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 P 41/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C 07 B 19/02; C 12 N 9/78, /80; C 12 P 13/00-/14, /20-/24, 41/00
US Cl	195: 2, 30, 50; 435: 106-110, 113 - 116, 128, 129, 227, 228

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	FR, A, 2 447 359 (AGENCE NATIONALE DE VALORISATION DE LA RECHERCHE) 22 August 1980	1-3,5,9,10,11
X	Chemical Abstracts, Vol 96 (1982) abstract No 160 701q, Adv Biotechnol 6th, 1980, 3, 227-33 (Fr)	1-3,9,11
A	EP, A, 0 093 782 (YAMADA) 16 November 1983	
A	US, A, 3 940 316 (AGENCE NATIONALE DE VALORISATION DE LA RECHERCHE) 24 February 1976	
A	US, A, 4 001 081 (AGENCE NATIONALE DE VALORISATION DE LA RECHERCHE) 4 January 1977	

* Special categories of cited documents: [14]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1986-09-02	1986 -09- 09
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